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A61P 1/04, 31/04Kim, Drummond [GB/GB]; Durness, Calver Road,  
Baslow, Derbyshire DE45 1RR (GB). LIU, Zhong-Ping  
[GB/GB]; 33 Greystones Crescent, Sheffield S11 7JN  
(GB).

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(74) Agents: BANNERMAN, David, Gardner et al.; Withers &amp; Rogers, Goldings House, 2 Hays Lane, London SE1 2HW (GB).

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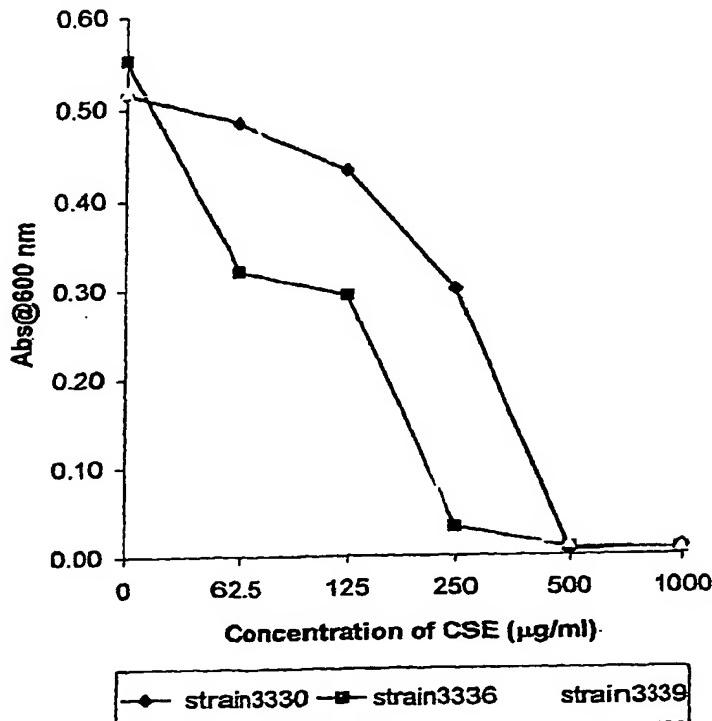
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0211943.6 23 May 2002 (23.05.2002) GB(71) Applicant (for all designated States except US):  
**SHEFFIELD, HALLAM UNIVERSITY [GB/GB];**  
Research and Business Development Department, City  
Campus, Howard Street, Sheffield S1 1WB (GB).(72) Inventors; and  
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(54) Title: ANTI-HELICOBACTER ACTIVITY OF CELERY SEED EXTRACT

Effect of CSE crude extract on the growth of the strains (3330, 3336,  
3339) of *H.pylori***BEST AVAILABLE COPY**

WO 03/099303 A1



European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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## INTERNATIONAL SEARCH REPORT

Internal Application No	PCT/GB 03/02244
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A. CLASSIFICATION OF SUBJECT MATTER
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IPC 7 A61K35/78 A61P1/04 A61P31/04

According to International Patent Classification (IPC) or to both national classification and IPC
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B. FIELDS SEARCHED
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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
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WPI Data, PAJ, BIOSIS, EPO-Internal, MEDLINE, EMBASE
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C. DOCUMENTS CONSIDERED TO BE RELEVANT
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Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 40258 A (DAVIS CRAIG KENDALL CHARLES ;BUTTERS DESLEY ETHEL (AU); INT CELERY) 13 July 2000 (2000-07-13) page 3, line 21 - line 33; examples 1-7 -----	2-4
X	US 6 352 728 B1 (DAVIS CRAIG KENDALL CHARLES ET AL) 5 March 2002 (2002-03-05) claim 1; examples 1-7 -----	2-4
X	WO 95 00157 A (MOBIUS CONSULTANCY PTY LTD ;DAUNTER BRIAN (AU)) 5 January 1995 (1995-01-05) page 7, line 23 - line 32 page 9, line 36 -page 10, line 4 ----- -----	2-4

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- \*&\* document member of the same patent family

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## Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax. (+31-70) 340-3016

Authorized officer

Pilling, S

## INTERNATIONAL SEARCH REPORT

Internat Application No  
PCT/GB 03/02244

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SINGH A ET AL: "Hepatoprotective activity of <i>Apium graveolens</i> and <i>Hygrophila auriculata</i> against paracetamol and thioacetamide intoxication in rats." JOURNAL OF ETHNOPHARMACOLOGY. IRELAND 15 DEC 1995, vol. 49, no. 3, 15 December 1995 (1995-12-15), pages 119-126, XP001118471 ISSN: 0378-8741 abstract	2-4
X	MOMIN RAFIKALI A ET AL: "Mosquitocidal, nematicidal, and antifungal compounds from <i>Apium graveolens</i> L. seeds." JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY, vol. 49, no. 1, January 2001 (2001-01), pages 142-145, XP001118472 ISSN: 0021-8561 abstract	2-4

## INTERNATIONAL SEARCH REPORT

Internal	Application No
PCT/GB 03/02244	

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
WO 0040258	A	13-07-2000	AU EP US WO	1811700 A 1140125 A1 2002081343 A1 0040258 A1		24-07-2000 10-10-2001 27-06-2002 13-07-2000
US 6352728	B1	05-03-2002	US	2002081343 A1		27-06-2002
WO 9500157	A	05-01-1995	AT AU AU WO CA CN DE DE EP ES IN JP NZ PL ZA	185698 T 691815 B2 6991094 A 9500157 A1 2165794 A1 1129908 A 69421280 D1 69421280 T2 0708651 A1 2140541 T3 178310 A1 9505024 T 267567 A 312714 A1 9404568 A		15-11-1999 28-05-1998 17-01-1995 05-01-1995 05-01-1995 28-08-1996 25-11-1999 13-07-2000 01-05-1996 01-03-2000 22-03-1997 20-05-1997 24-10-1997 13-05-1996 20-03-1995

### Anti-Helicobacter Activity of Celery Seed Components

The invention relates to the use of biologically active celery seed extracts to inhibit the growth and replication of the bacterium, *Helicobacter pylori*.

5

Arthritis and rheumatism are important world-wide problems. Around 1% of the UK population are affected at some stage in life. Complaints of this nature not only cause significant disability but may also have a severely detrimental effect on the psychological state of the sufferers. Conventionally these complaints are treated with

10 analgesic/antipyretic drugs and non-steroidal anti-inflammatory drugs (NSAIDs). However NSAIDs can have serious side effects, such as gastrotoxicity, causing for example gastric ulceration, and hence research has been made into alternative sources of anti-inflammatory drugs. In particular compounds extracted from higher plants have been considered. Lewis *et al* (1985) and Whitehouse *et al* (1999) found that the  
15 extracts of celery (*Apium graveolens*) (CSE) had significant anti-inflammatory activity in animal models with reduced adverse effects. A further risk factor in the pathogenesis of peptic ulcer disease is *H.pylori* infection. Chan (1997) found that eradication of *H.pylori* before NSAID therapy reduced the risk of ulcer development by about fourfold. PCT/US99/25873 discloses the use of celery seed extract for the prevention  
20 and treatment of pain, inflammation and gastrointestinal irritation.

The inventors have surprisingly found that components of celery seed extract may be used to control the growth of *Helicobacter pylori*.

25 The invention provides the use of celery seed or celery seed extract (CSE) for the inhibition of growth and replication of *Helicobacter pylori*.

A preferred CSE is produced by supercritical fluid extraction of the starting product. By CSE we mean a natural product derived from celery seed, or a pharmaceutical  
30 equivalent thereof. This is preferably an ethanol/water extract, especially 50% to 90%,

60% to 85%, most preferably an 80% Vol:Vol ethanol/water extract. The term includes the isolated compounds obtainable from CSE.

Preferably the active component of the celery seed extract is selected from the group:

- 5     3-n-butyl 4,5-dihydrophthalide, 3-n-butyl phthalide,  $\alpha$ -Eudesmol,  $\beta$ -Eudesmol diethyl phthalate and cis, cis-9,12-Octadecadienoic acid.

The invention further provides a pharmaceutical composition for the inhibition of growth and replication of *Helicobacter pylori*, comprising celery seed extract.

- 10   Also provided is the use of celery seed or celery seed extract in the preparation of a pharmaceutical composition for the treatment of *Helicobacter pylori* infection.

Preferably the *H.pylori* infection is in a mammal, such as a human. Preferably the infection is within the digestive tract, especially the stomach of the mammal.

- 15   The pharmaceutical composition may be administered orally, e.g. in the form of an oral suspension, solution or tablet. Dosages may be 300-2000 mg. daily in divided doses preferably or even higher.

The pharmaceutical composition may comprise one or more pharmaceutically acceptable carriers, bulking agents or excipients known in the art (e.g. in the form of a tablet or injectable solution).

- 20   A further aspect of the invention provides celery seed or celery seed extract for use in the manufacture of a medicament to treat a *Helicobacter pylori* infection.

The invention will now be described in detail with reference to the figures in which:

Table 1 shows the effect of the crude extract of CSE on the growth of different strains (3330, 3336 and 3339) of *H.pylori*.

- 25   Table 2 shows the distribution of antimicrobial activity against *H. pylori* (strain 3339) in the crude extract and different fractions of CSE.

Table 3 shows antimicrobial activity of the subfractions from pet. ether fraction against *H. pylori* (strain 3339).

Table 4 shows antimicrobial activities of compounds from subfractions 6 and 10 against *H. pylori* (strain 3339).

5 Fig.1 shows the effect of CSE crude extract on the growth of the strains (3330, 3336, 3339) of *H.pylori*

Fig.2 shows the bioassay-guided fractionation scheme of celery seed extract (antimicrobial agents enclosed in boxes).

10 Fig.3 shows the antimicrobial activity of pet. ether fraction and subfractions 6 and 10 against *H.pylori* (strain 3339).

Fig.4 shows the analytical separation of mixture from subfraction 10. Column: Nucleosil® C18, 250 x 4.6 mm. I.D.; Mobile phase: ACN/water (60:40); Flow rate: 1.0 m./min; Detection: UV @ 236 nm; Injection volume: 10  $\mu$ g in 1 ml of 40% ACN in water ; Temperature: Ambient; ATT:3.

15 Fig.5 shows the antimicrobial activities of compounds against *H.pylori* (strain 3339)

Fig.6 shows the EI-MS spectrum of compound 6-1

Fig.7 shows the  $^1\text{H}$  NMR spectrum of compound 6-1

Fig.8 shows the  $^{13}\text{C}$  NMR spectrum of compound 6-1

Fig.9 shows the EI-MS spectrum of compound 6-1

20 Fig.10 shows the EI-MS spectrum of compound 6-3

Fig.11 shows the EI-MS spectrum of compound 6-4

Fig.12 shows the EI-MS spectrum of compound 10-1

### Antimicrobial test

#### Bacterial strains

Three strains of *H.pylori* (3330, 3336 and 3339) isolated from British patients with gastric ulcer (duodenal ulcer or gastritis) were studied. The identities of *H. pylori* were confirmed by Gram stain and urease reaction. The bacteria were stored at -80°C in aliquots of 1ml of brocella broth containing 15% (v/v) glycerol (Kitsos and Stadtlander, 1998).

#### Celery seed extract (CSE)

Test CSE was provided as dark green highly viscous liquid (supplied by Beagle International Pty. Ltd, Nerang, Qld., Australia). Initially CSE was dissolved in dimethylsulfoxide (DMSO) as stock solution (~100mg/ml, final DMSO concentration in cultures ≤1%).

#### Media

For the Brucella broth (BB), (BBL, USA), Brucella (28g) was added to 1L of distilled water. After the medium was autoclaved at 120°C for 15 mins, fetal bovine serum (50 ml) was added (Morgan *et al*, 1987).

#### Inocula

Thawed isolates were inoculated onto chocolate agar plates (Mérieux) and incubated under microaerophilic conditions (85%N<sub>2</sub>, 10%CO<sub>2</sub>, 5%O<sub>2</sub>) for 48 h at 37°C. Colonies were suspended in 5ml of Brucella broth and adjusted to a turbidity equivalent to a No.2 McFarland standard (approximately 6x10<sup>8</sup> CFU/ml) for broth dilution method. The final inoculum was 10<sup>7</sup> CFU/ml for agar dilution method by a further 50-fold dilution.

#### Broth dilution test

The CSE suspension (1mg/ml) was serially two-fold diluted in BB. The concentrations (1000, 500, 250, 125, and 62.5 µg/ml) were obtained. The solutions were added to the

column wells of 24-well plate each in equal volume (1ml/well). 20 $\mu$ l of cell suspension was inoculated into each row wells of 24-well plates (except last row wells). The culture dishes were gently agitated following the addition of the inoculum and then placed at 37°C under microaerophilic conditions for three days. At the end of 5 incubation, 1ml of bacterial culture solution from each well were diluted to one in a million dilution (10<sup>-6</sup>). Then 20  $\mu$ l aliquots from each solution were transferred to columbia agars and incubated for an additional three days. Generally, only spots with between 7-11 colonies were counted. Growth was determined on the basis of calculating the number of bacteria per millilitre (numbers of bacteria/ml = numbers of 10 colonies on plate  $\times$  reciprocal of dilution of sample). Bacteria growth, culture medium and extract controls were run in parallel. (Osato *et al.*, 1999).

#### **Chromatographic Methods**

Column chromatography was performed on silica gel 60 (40-60  $\mu$ m, Merck). Analytical thin layer chromatography (TLC) was carried out on precoated silica gel 60 15 F<sub>254</sub> plates (layer thickness 0.2 mm, Merck), developed with the following solvent, hexane-EtOAc (70:30), chloroform-methanol (98: 2). For isolation monitoring, spots were located by their absorption under ultraviolet (UV) light (254 and 366 nm) directly. After that the plates were sprayed with anisaldehyde reagent and heated at 110°C for 5 min (Dey and Harborne, 1991).

20 **HPLC (1090 LC, Hewlett Packard, UK) analytical and semi-preparative purification**

#### Analytical conditions:

Analytical column: Nucelosil® C18, particle size 5 $\mu$ m, 250  $\times$  4.6 mm I.D., catalogue No.89141 (Alltech, Carnforth, Lancashire, UK)

25 Mobile phase: acetonitrile/water (60:40)

Flow rate: 1.0 ml/min

Injection volume: 10 $\mu$ l

Detection: UV @ 236 nm

Sample: mixture of compounds 10-2, 10-3 and 10-4 (Conc.= 500 µg/ml)

Temperature: ambient

ATT: 3

Semi-preparative conditions:

- 5      Semi-preparative column: Luna C18(2), particle size 5µm, 250 x 10.00 mm I.D., catalogue No.00G-4252-NO (Phenomenex, Macclesfield, Cheshire, UK)

Mobile phase: acetonitrile/water (60:40)

Flow rate: 5.0 ml/min

Injection volume: 100µl

- 10     Detection: UV @ 236 nm

Sample: mixture of compounds 10-2, 10-3 and 10-4 (Conc. = 5mg/ml)

Temperature: ambient

ATT: 6

- 15     Spectroscopic Methods

Mass spectrometry (MS)

The Mass spectra were recorded on a VG 70/70 Sector Mass Spectrometer instrument (Micromass, Manchester, UK) in the Laboratory of Biomedical research centro (Sheffield Hallam University).

- 20     Nuclear magnetic resonance (NMR)

NMR spectra were recorded in CDCl<sub>3</sub> at RT on a Bruker Unity Ac 250 MHz (<sup>1</sup>H 250MHz; <sup>13</sup>C, 62.9 MHz).

**Results and Discussion**

25

The 80% ethanol extract exhibited appreciable antimicrobial activity at the minimum inhibitory concentrations (MIC) of 250, 125 and 125µg/ml, respectively, against *H.*

*pylori* strains 3330, 3336 and 3339. The results of antimicrobial activity of CSE are given in Table 1 and Fig.1. The bioassay-guided fractionation scheme of CSE is illustrated in Fig.2. The fractionation for the isolation of the active compounds was performed from the 80% ethanol extract of CSE. The susceptibility of *H. pylori* strain 3339 was higher than 3330 and 3336. Later, in antimicrobial activity testing of fractions and subfractions of CSE, only *H. pylori* 3339 strain was chosen for fractionation guide. The residue of 80% ethanol extract of CSE was subsequently successively partitioned with organic solvents and water. The activity emerged predominantly in the petroleum ether layer ( $MIC = 15.625 \mu\text{g/ml}$ ) as compared to the other solvents, diethyl ether ( $MIC=125\mu\text{g/ml}$ ), ethyl acetate ( $MIC > 500 \mu\text{g/ml}$ ) and water ( $MIC > 500 \mu\text{g/ml}$ ) (Table 2).

The petroleum ether fraction was directly subjected to column chromatography on silica gel with hexane, hexane-EtOAc (99:1), hexane-EtOAc (95:5), hexane-EtOAc (70:30) and EtOAc as eluent. Fractions with the same retardation factors were combined to yield 11 major fractions. Each subfraction was tested for antibacterial activity against *H. pylori*. The results of the antimicrobial testing of the different subfractions are shown in Table 3. The most pronounced antimicrobial activity successively resided in the subfraction 6 eluted with hexane-EtOAc (95:5) ( $MIC = 15.625 \mu\text{g/ml}$ ) and the subfraction 10 eluted with hexane-EtOAc (70:30) ( $MIC = 15.625 \mu\text{g/ml}$ ) (Fig.3). Subfraction 6 was further purified by silica gel column chromatography (hexane-ether, 10:1, as solvent) and preparative TLC using chloroform/pet. ether (3:1) to yield compounds 6-1, 6-2, 6-3 and 6-4. Subfraction 10 was further purified with hexane-ether (7:3) as mobile phase to afford a pure compound 10-1 and a mixture. The mixture was dissolved in 40% ACN in water and passed through the DPA-6S SPE column (Supelco, UK) to remove the chlorophyll. The eluate with methanol was evaporated to dryness and reconstituted in 40% ACN in water for HPLC analysis. It was separated into three compounds 10-2, 10-3 and 10-4 by analytical HPLC using ACN/water (60:40) as mobile phase (Fig.4). Large quantity of individual pure compounds will be obtained by semi-preparative HPLC and sent for MS and NMR spectroscopic analysis.

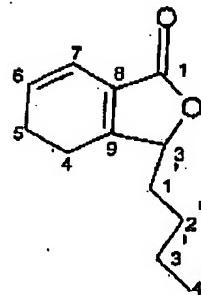
Compounds 6-1, 6-2, 6-3, 10-1 and the combination of 6-1 and 6-3 were evaluated for antimicrobial activity. The results indicated they were partly responsible for the antimicrobial activity of CSE (Table 4 and Fig.5). The mixture of 6-1 and 6-3 by different combination did not exert a synergistic effect in antimicrobial activity. The mixture of compounds 10-2, 10-3 and 10-4 showed an interesting antimicrobial activity against *H. pylori*. Very recently, Momin and Nair (2001) isolated and characterized three bioactive compounds, sedanolide, senkyunolide-N and senkyunolide-J from CSE with the significant mosquitocidal, nematicidal and antifungal activities. Further study will confirm with MS and NMR data if compounds 10-2, 10-3 and 10-4 are corresponding to sedanolide, senkyunolide-N and sekyunolide-J. The antimicrobial activity of individual compound will be tested as well.

The exact structures are confirmed by comparison of their physical and spectral data ( $[\alpha]$ ,  $^1\text{H}$  and  $^{13}\text{NMR}$ ) with data in the literature. Structural elucidation of the compounds isolated from active fractions 6 and 10 are given below:

Compound 6-1 was obtained as pale yellow oil with a distinct celery odour. The electron impact mass spectrometry (EI-MS) spectrum (Fig.6) of the compounds showed the molecular ion peak at mass/charge ratio (m/z) 192 (composition, 22.9%), corresponding to the molecular formula  $\text{C}_{12}\text{H}_{16}\text{O}_2$ . Other major peaks were at m/z (composition, %) 163 (3.6), 135 (5.3), 108 (21.7), 107 (100%), 85 (9.7), 79 (24.3), 77 (24.2) and 57 (14.4).

The  $^1\text{H}$  NMR spectrum (Fig.7) displayed a doublet at 6.12 ppm (1H,  $J=10$  Hz) and a multiplet at 5.9 ppm for the vinyl protons, H-7 and H-6, respectively, as well as multiplet at 4.9 ppm for H-3. In  $^{13}\text{C}$  NMR spectrum (Fig.8), the signals at 128.4, 116.8 and 124.5 ppm were consistent with disubstituted and tetrasubstituted double bands composed of C-6, C-7 and C-1a, C-3a, respectively. In addition, tetra substituted signals appeared for the side chain (C-1', C-2', C-3', C-4') in the range of 13.8-22.4 ppm. The signals due to C-1, C-4 and C-5 appeared at 161, 31.9 and 26.7 ppm.

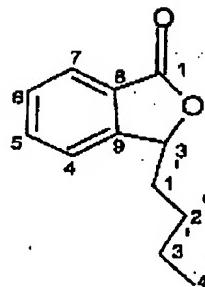
On the basis of EI-MS and  $^1\text{H}$ - and  $^{13}\text{C}$ - NMR, compound 6-1 was identified as 3-n-butyl 4,5-dihydrophthalide (sedanenolide) (Bjeldanes and Kim, 1977).



Experimental data

- 10 Compound 6-1 EI-MS: m/z 192.3 (calculated for  $C_{12}H_{16}O_2$ ).  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  0.9 (t, 3H, J= 7.2, H-4'), 1.2-1.8 [m, 6H, H1(1',2',3')], 2.45 (m, H-4,5), 4.9 (m, 1H, H-3), 5.9 (m, 1H, H-6), 6.2 (d, 1H, J=10, H-7);  $^{13}C$  NMR ( $CDCl_3$ ):  $\delta$  13.8-22.4 (C-1', 2', 3', 4'), 26.7-31.8 (C-4,5), 82.5 (C-3), 116.8 (C-7), 128.3 (C-6), 124.5-135 (C-8, 9), 161.4 (C-1).
- 15 Compound 6-2 was obtained as pale yellow oil with a distinct celery colour. The EI-MS spectrum (Fig.9) of 6-2 showed the molecular ion peak as mass/charge ratio (m/z) 190, corresponding to the molecular formula  $C_{12}H_{14}O_2$ . Other major peaks were at m/z 163, 148, 144, 133 (100%), 115, 105, 91 and 77.
- On the basis of EI-MS and  $^1H$ - and  $^{13}C$ - NMR, compound 6-2 was identified as  
20 3-n-butyl phthalide (Zheng *et al*, 1993).

5



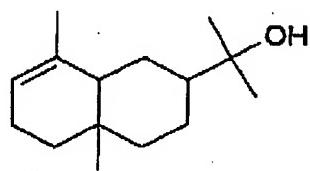
Experimental data

10 EI-MS: m/z 190.2 (calculated for  $C_{12}H_{14}O_2$ ).  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  0.85 (t, 3H J=7.1, H-4'), 1.2-2.10 [m, 6H, H-(1' 2', 3')], 5.42 (dd, 1H, J=7.8 and 4.1 Hz, H-3), 7.39 (d, 1H, J=7.5, H-4), 7.46 (t, 1H, J=7.5, H-6), 7.62 (t, 1H, J=7.5 Hz, H-5), 7.83 (d, 1H, J=7.5 Hz, H-7);  $^{13}C$  NMR ( $CDCl_3$ ):  $\delta$  14.08 (C-4'), 22.65 (C-3'), 27.01 (C-1'), 34.62 (C-2'), 81.75 (C-3), 121.68 (C-4), 125.57 (C-6), 125.96 (C-9), 128.94 (C-7), 134.20 (C-5), 150.02 (C-8), 171.04 (C-1).

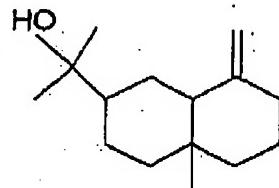
15 (Large quantity of 6-2 will be obtained by purification using PTLC or semi-preparative HPLC, then  $^1H$  NMR and  $^{13}C$  NMR will be acquired again to get clear spectra).

For compound 6-3, the EI-MS spectrum (Fig.10) showed the molecular ion peak at mass/charge ratio (m/z) 222, corresponding to the molecular formula  $C_{15}H_{26}O$ . Other major peaks were at m/z 204, 189, 162, 149, 135, 109, 108, 95, 81, 59 and 41. On the 20 basis of EI-MS, the compound 6-3 was identified as mixture of  $\alpha$  and  $\beta$ -Eudesmol (El-Sayed *et al.* 1989).

$^1H$  NMR and  $^{13}C$  NMR spectra will confirm the structure of 6-3. But there is not enough sample by now for measuring  $^1H$  NMR and  $^{13}C$  NMR (around 10-20 mg needed). The possible structure of compound 6-3 is as below:



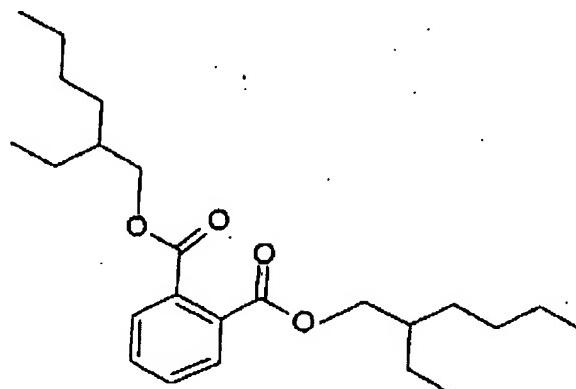
5

 $\alpha$ -Eudesmol $\beta$ -Eudesmol

Compound 6-4 was obtained as colourless oil. The EI-MS spectrum of 6-4 (Fig.11) showed the major peaks at m/z 279, 167, 149, 83, 71, 57 and 43. On the basis of  
10 EI-MS, the Compound 6-4 was identified as dioctyl phthalate, corresponding to the molecular formula  $C_{24}H_{38}O_4$  (MW = 390.54) (MS library).

$^1H$  NMR and  $^{13}C$  NMR spectra will confirm the structure of 6-4. But there is not enough sample by now for measuring  $^1H$  NMR and  $^{13}C$  NMR (around 10-20 mg needed). The possible structure of compound 6-4 is as below:

15



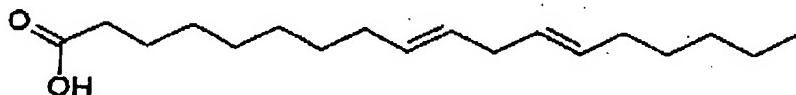
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Compound 10-1 was obtained as a colourless oil. The EI-MS spectrum (Fig.12) of 10-1 showed the molecular ion peak at mass/charge ration (m/z) 280, corresponding to the molecular formula  $C_{18}H_{32}O_2$ . Other major peaks were at m/z 137, 123, 109, 95, 81, 67, 55, 54 and 41. On the basis of EI-MS, the compound 10-1 was identified as linoleic acid (cis, cis - 9,12- Octadecadienoic acid) (MS library).

5        $^1H$  NMR and  $^{13}C$  NMR spectra will confirm the structure of 10-1. But there is not enough sample for measuring  $^1H$  NMR and  $^{13}C$  NMR (around 10-20 mg).

The possible structure of compound 10-1 is as below:

10



### Conclusion

Overall the CSE has shown interesting antimicrobial activity against *H. pylori*. Five compounds have been purified which are partly responsible for the antimicrobial properties. The structure elucidation of compounds is still undergoing. Further work will continue to purify the active constituents in subfraction 10 and other subfractions and to test the anti-cytokine activity and cartilage protection properties. If the compounds from subfractions 6 and 10 are not responsible for the anti-inflammatory activity, the constituents maybe reside in other fractions and subfractions.

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**Table 1.** Effect of the crude extract of CSE on the growth of different strains (3330, 3336 and 3339) on *H. pylori*.

Strains	MIC ( $\mu\text{g/ml}$ )	MBC ( $\mu\text{g/ml}$ )
3330	250	500
3336	125	500
3339	125	500

**Table 2** Distribution of antimicrobial activity against *H. pylori* (strain 3339) in the crude extract and different fractions of CSE.

Fractions	MIC ( $\mu\text{g/ml}$ )	MBC ( $\mu\text{g/ml}$ )
Crude extract	125	500
Pet. ether	15.625	31.25
Diethyl ether	125	500
Ethylacetate	>500	>500
Water	>500	>500

**Table 3** Antimicrobial activity of the subfractions from pet. ether fraction against *H.pylori* (strain 3339).

Fractions and subfractions	MIC ( $\mu\text{g/ml}$ )
Pet. ether	15.625
Sub-1	>125
Sub-2	>125
Sub-3	125
Sub-4	62.5
Sub-5	62.5
Sub-6	15.625
Sub-7	31.25
Sub-8	31.25
Sub-9	62.5
Sub-10	15.625
Sub-11	31.25

**Table 4** Antimicrobial activities of compounds from subfractions 6 and 10 against *H.Pylori* (strain 3339).

Compounds	MIC ( g/ml)	MBC ( g/ml)
sedanenolide	31.25	62.5
3-n Butyl phthalide	15.625	N.T.
Eudesmol	15.625	125
Eudesmol + sedanenolide (major) (minor)	15.625	N.T.
Eudesmol + sedanenolide (minor) (major)	31.25	N.T.
Linoleic acid	62.5	>125
10-2, 10-3 and 10-4	12.5	25

N.T. : not tested

**Claims**

1. Use of celery seed or celery seed extract (CSE) for the inhibition of growth and replication of *Helicobacter pylori*.
- 5 2. Celery seed or celery seed extract for the preparation of a pharmaceutical composition to treat *Helicobacter pylori* infection.
- 10 3. Use according to claim 1 or claim 2 wherein the celery seed extract is an ethanol/water extract.
- 15 4. Use according to any preceding claim wherein the active component of the celery seed extract is selected from 3-n-butyl 4,5-dihydrophthalide, 3-n-butyl phthalide,  $\alpha$ -Eudesmol,  $\beta$ -Eudesmol dioctyl phthalate and cis, cis-9,12-Octadecadienoic acid.
5. A method of treating *Helicobacter pylori* infection comprising administering a pharmaceutically effective amount of celery seed or a celery seed extract

**ABSTRACT****Anti-Helicobacter Activity of Celery Seed Components**

- 5 The application discloses celery seeds or celery seed extracts for treating *Helicobacter pylori* infections.

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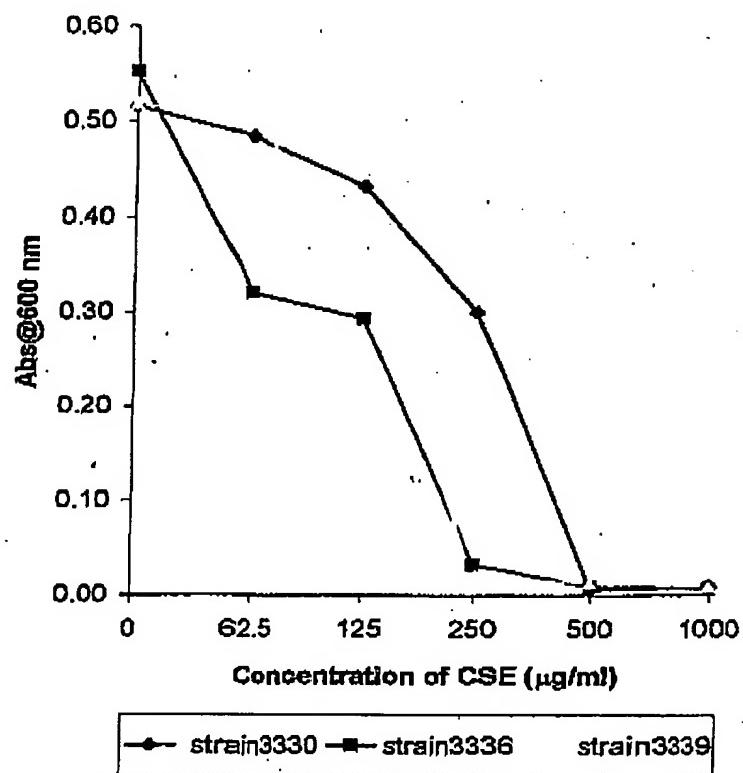


Fig.1 Effect of CSE crude extract on the growth of the strains (3330, 3336, 3339) of *H.pylori*

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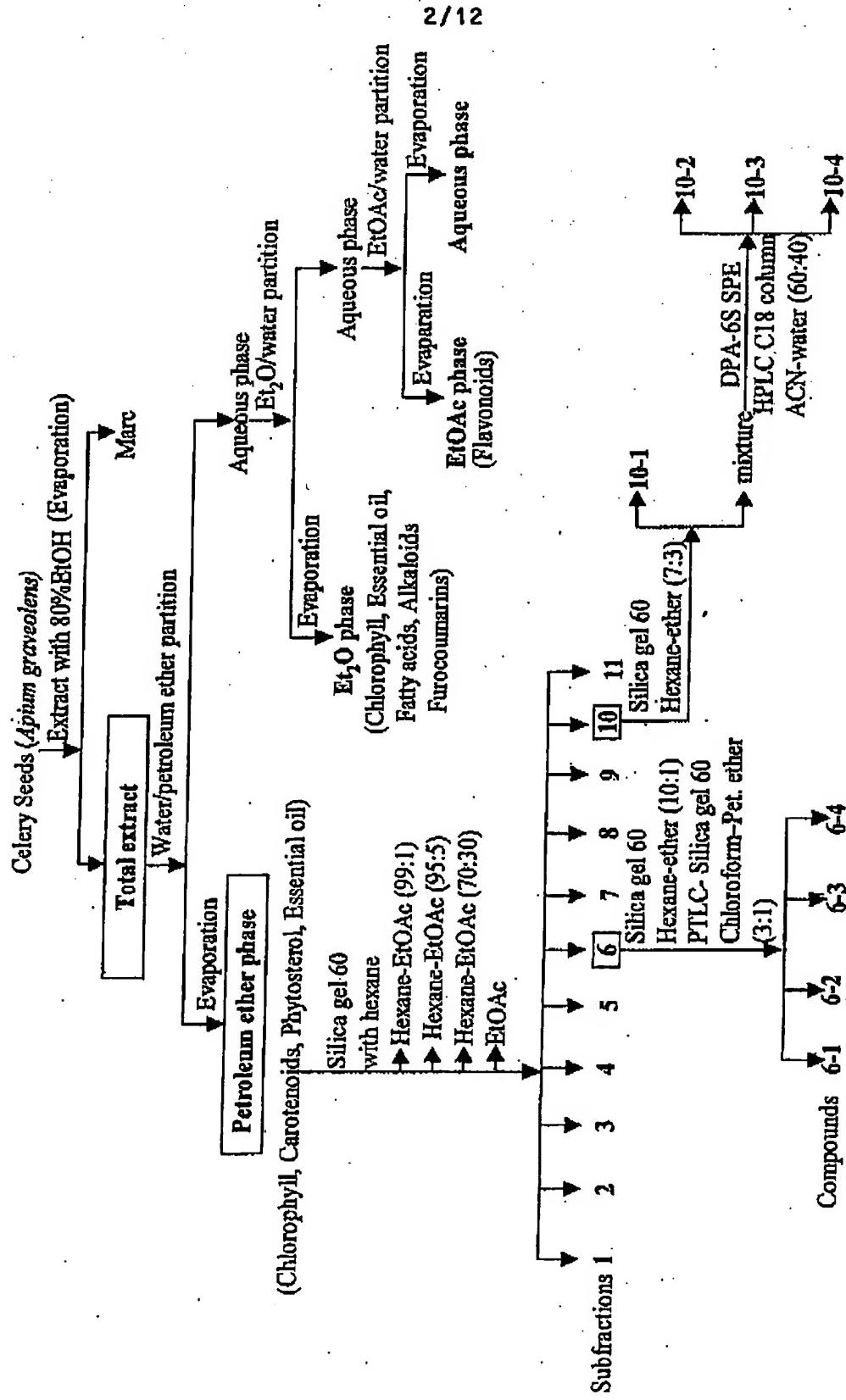


Fig.2 Bioassay-guided fractionation scheme of celery seed extract (antimicrobial agents enclosed in boxes)

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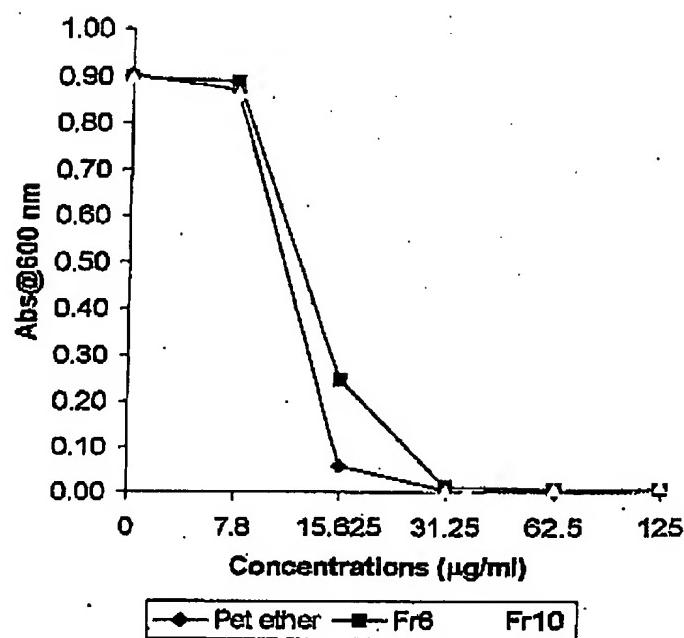


Fig.3 Antimicrobial activity of pet.ether fraction and subfractions 6 and 10 against *H.pylori* (strain 3339)

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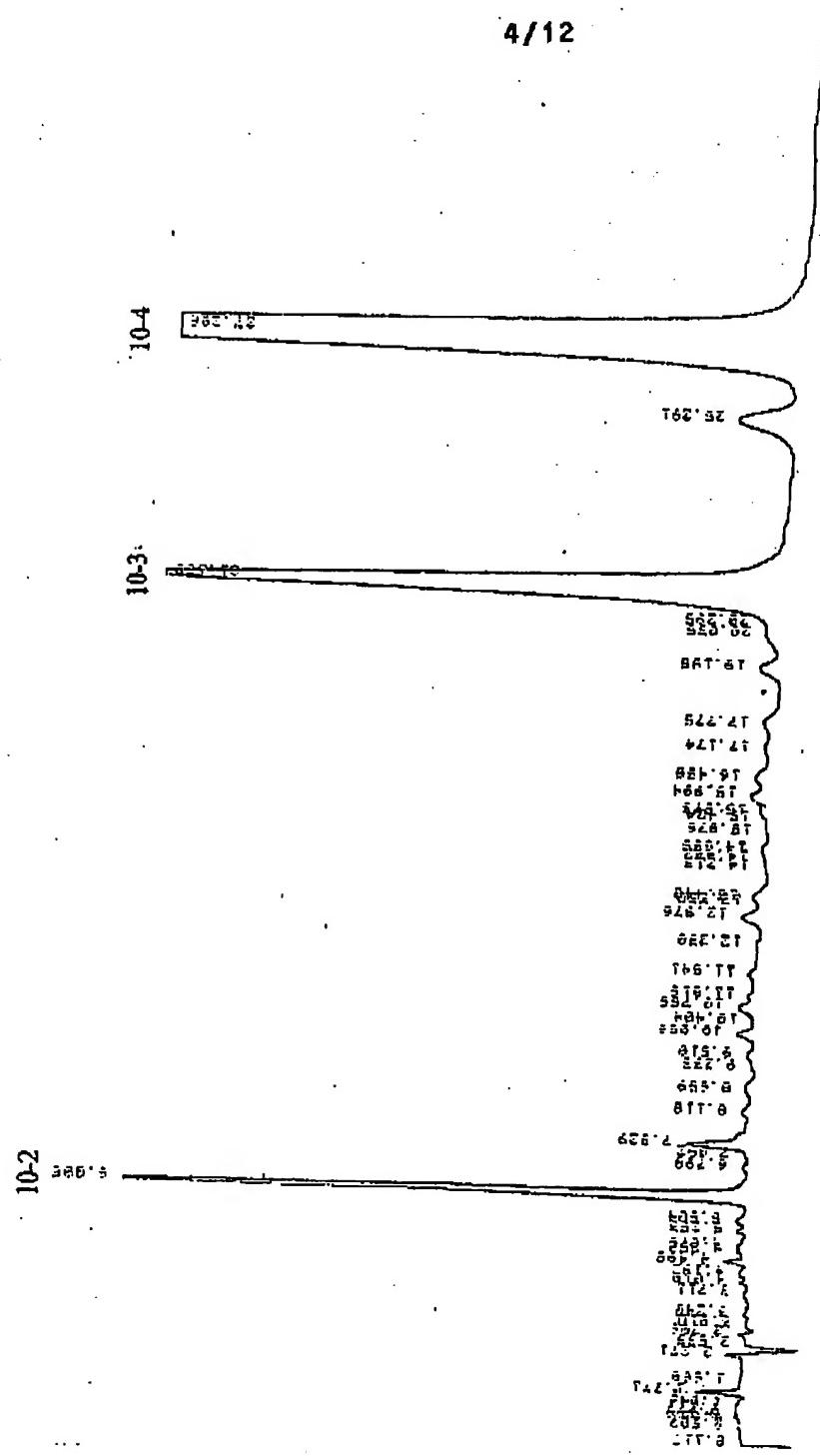


Fig.4 Analytical separation of mixture from subfraction 10. Column: Nucleosil<sup>®</sup> C18, 250 x 4.6 mm I.D.; Mobile phase: ACN/water (60:40); Flow rate: 1.0 ml/min; Detection: UV @ 236 nm; Injection volume: 10  $\mu$ l; Sample: 500  $\mu$ g in 1 ml of 40% ACN in water; Temperature: Ambient; ATT: 3.

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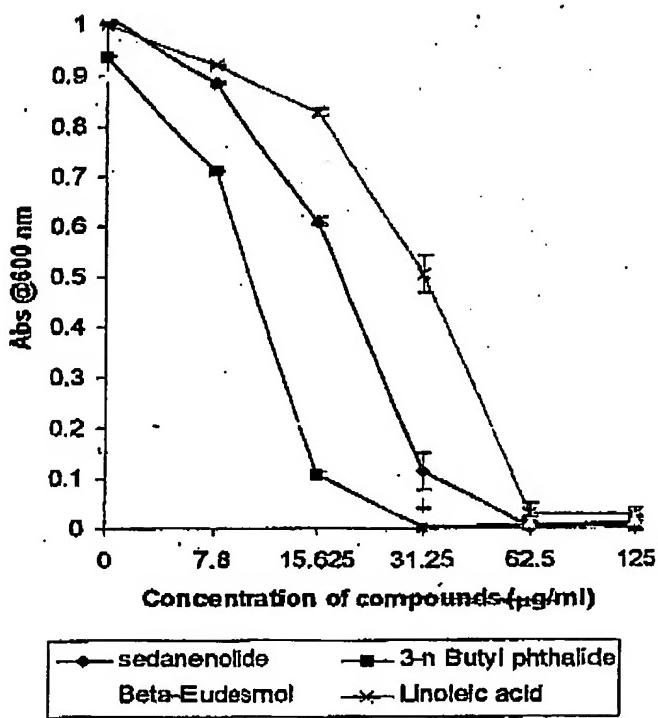
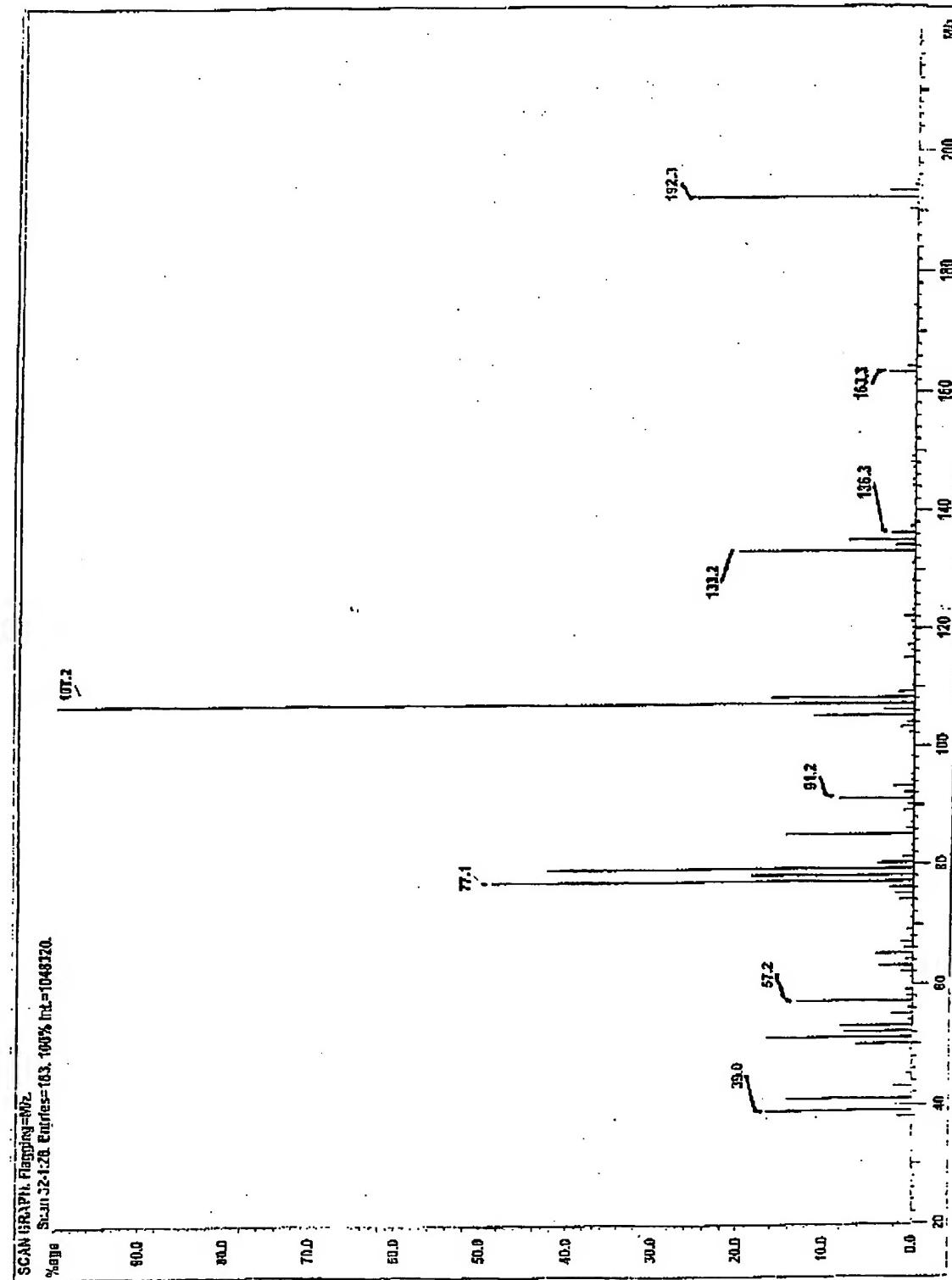


Fig.5 Antimicrobial activities of compounds against *H. pylori* (strain 3339)

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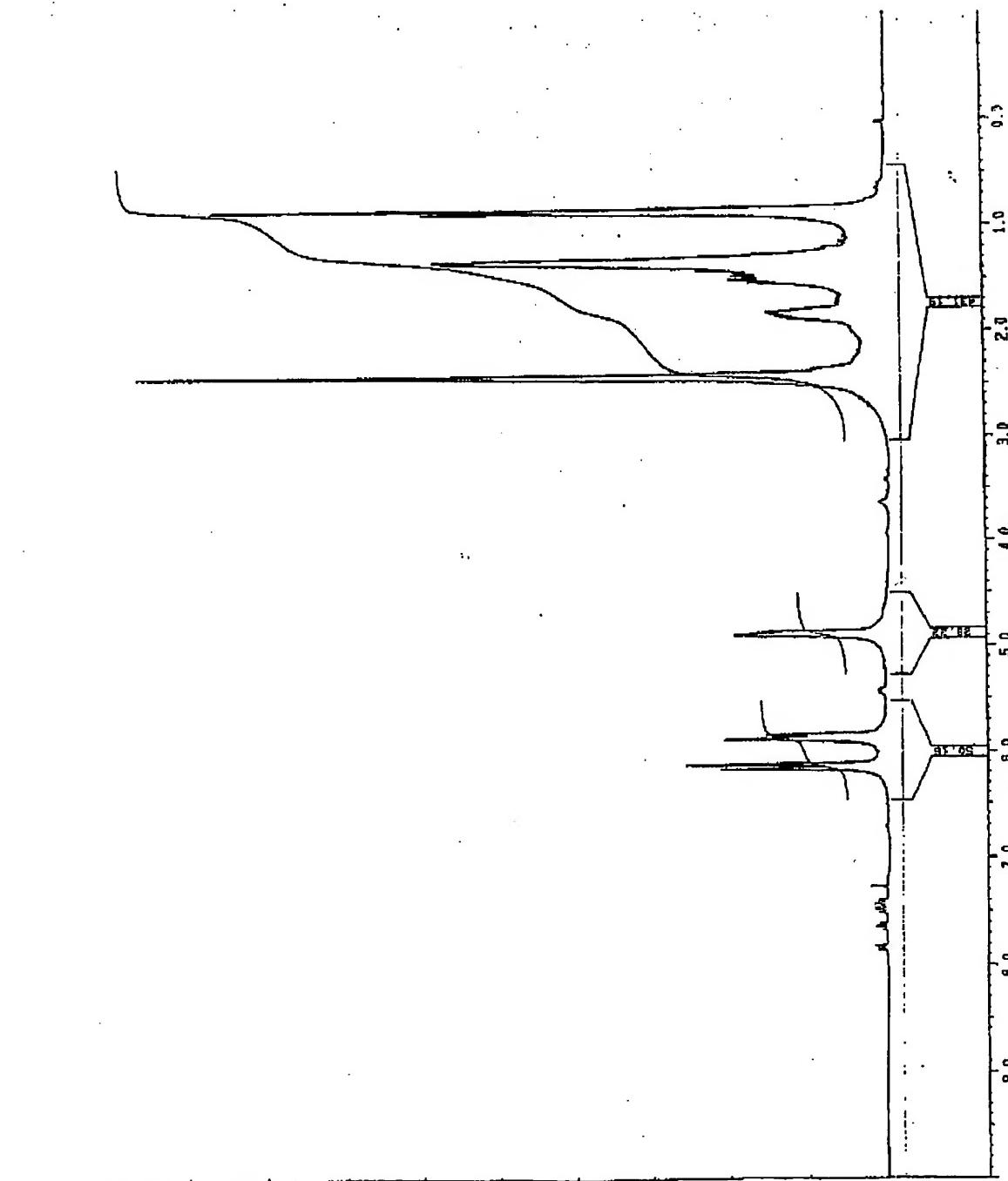
Fig.6 EI-MS spectrum of compound 6-1



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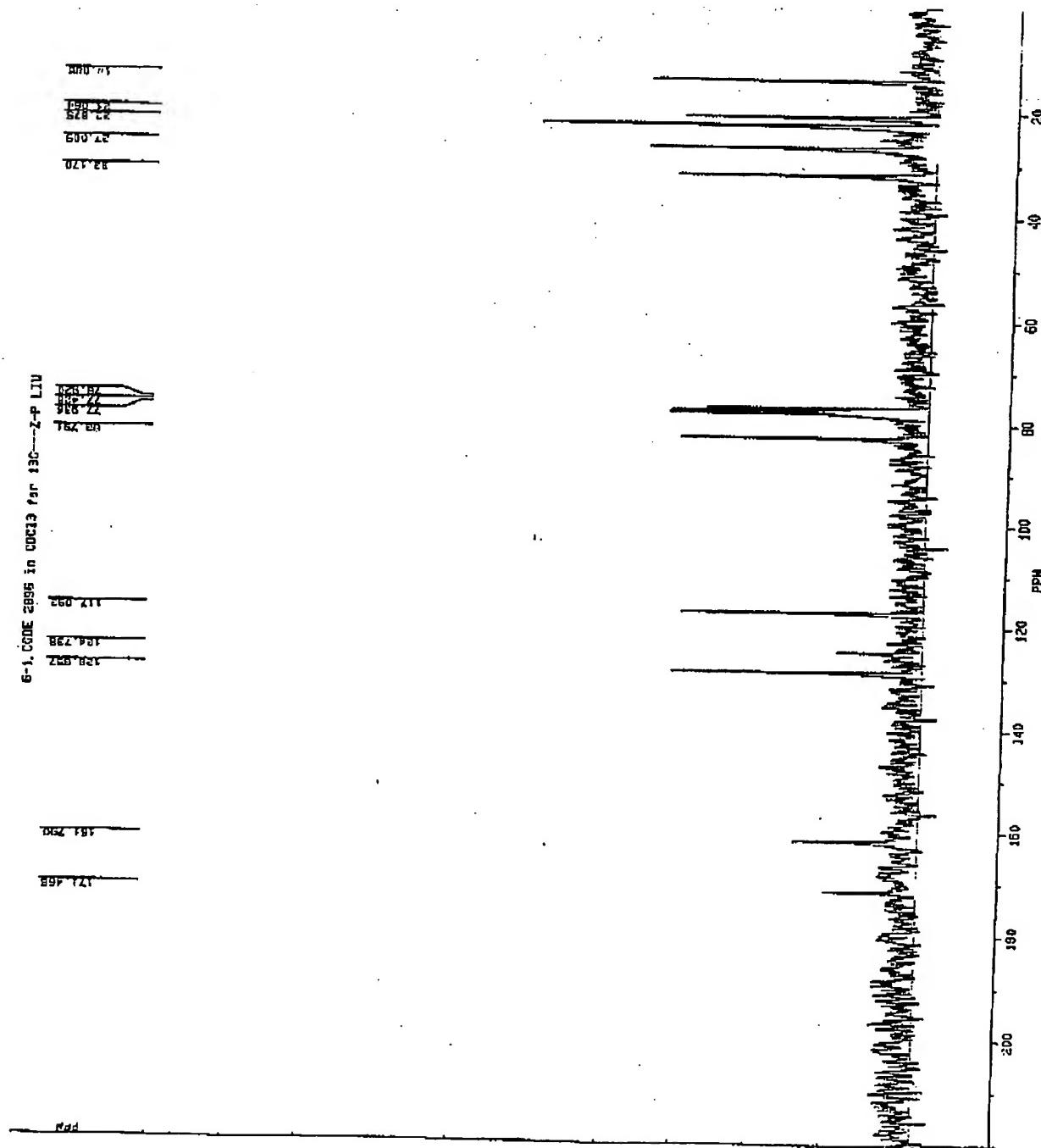
Fig. 7  $^1\text{H}$  NMR spectrum of compound 6-1



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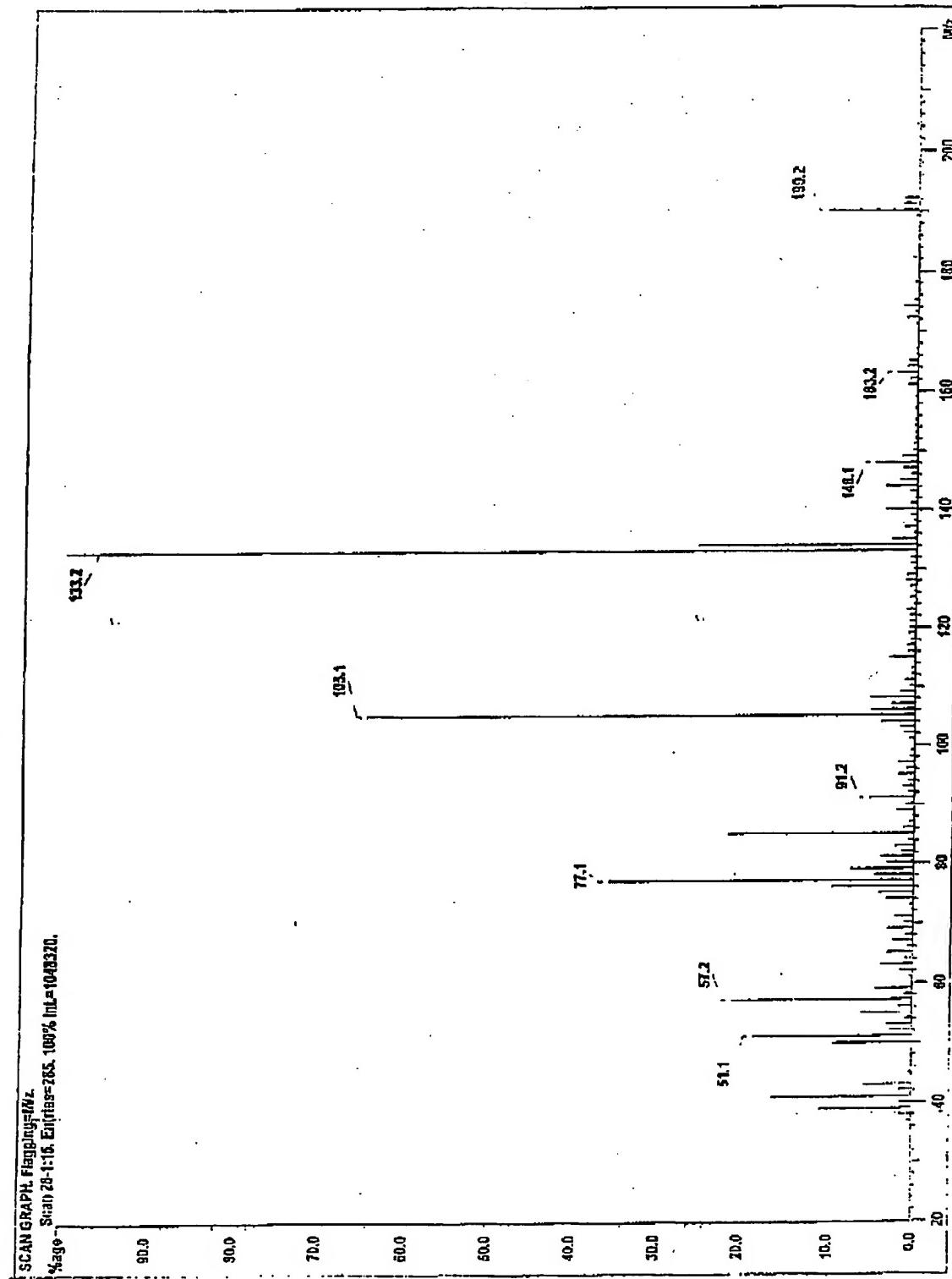
**Fig.8**  $^{13}\text{C}$  NMR spectrum of compound 6-1



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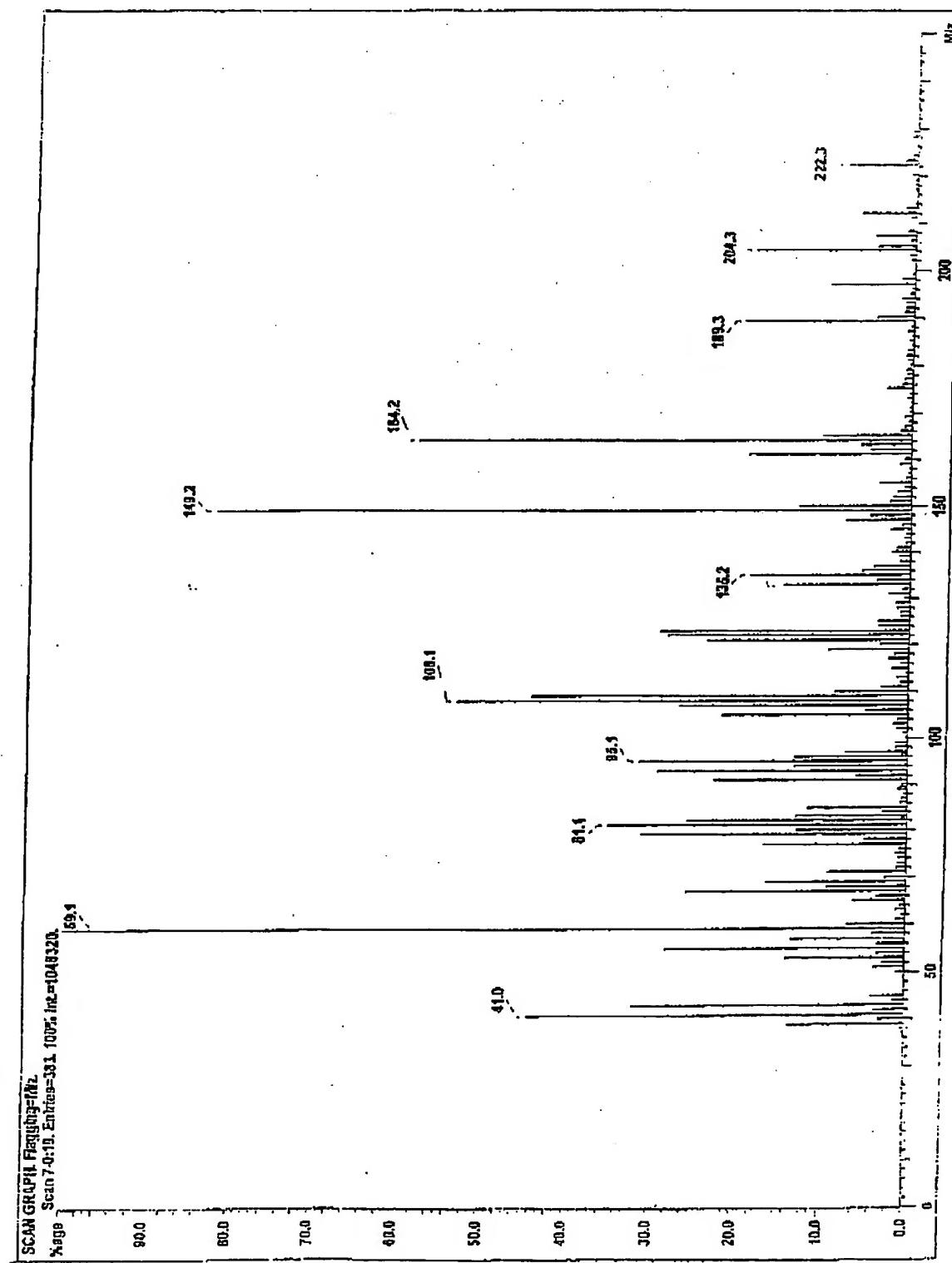
Fig.9 EI-MS spectrum of compound 6-2

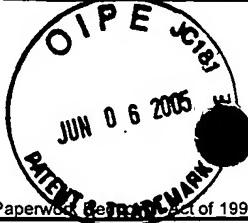


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Fig.10 EI-MS spectrum of compound 6-3





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First Named Inventor	RAINSFORD, Kim D.
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		EL-SAYED, et al., Int. J. Crude Drug Res., 1989, 27(4):185-88.	
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		MOMIN, et al., J. Agric. Food Chem., 2001, 49(1):142-45.	
		MORGAN, et al., J. Clinical Microbiology, 1987, 25(11):2123-25.	
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June 3, 2005

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Jill T. Powlick  
Attorney Registration No.: 42,088  
One American Square, Box 82001  
Indianapolis, Indiana 46282-0200  
Telephone: (317) 236-2100

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